

Original Article

Exploration of the effect of probiotics supplementation on intestinal microbiota of food allergic mice

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Abstract: Environmental factor-induced alterations in intestinal microbiota have been demonstrated to be associated with increasing prevalence of food allergy. However, it is not clear to what extent oral administration of probiotics can affect gut microbiota composition, thus inhibiting food allergy development. Using ovalbumin (OVA)-sensitized murine model, it was demonstrated that probiotics ameliorated allergic symptoms, including reducing OVA specific-IgE, and -IgG1 levels in the serum, Th2 cytokines release in spleen, and occurrence of diarrhea. Moreover, 16S rRNA analysis showed that the probiotics-mediated protection was conferred by an enrichment of *Coprococcus* and *Rikenella*. The present study supports the theory that probiotics can treat food allergy by modulating specific genera of the gut microbiota.

Keywords: Intestine, microbiota, food allergy, OVA, 16S rRNA

Introduction

Food allergy is an adverse immune response to certain kinds of food. It is estimated that food allergy affects about 8% of children and 4% of adults [1, 2]. The rapid increase in the prevalence of food allergy over past several decades cannot be explained by genetic variation alone. In current, avoidance of dietary allergens is the only proven remedy available for food allergic suffers.

Growing evidence suggests that gut microbiota exerts profound influence on immune system maturation and tolerance acquisition. Intestinal microflora alteration, caused by environmental factors (e.g., mode of birth, antibiotics, diet, vaccination, sanitation), has been observed to be associated with many gastrointestinal diseases, including food allergy [3], inflammatory bowel diseases [4], or colorectal cancer [5-8]. Of note, intestinal microflora has been demonstrated to play an important role in maintaining the Th1/Th2 balance [9], which is the key mechanism involved in allergic diseases.

The role of probiotics in allergic disease has been highlighted recently. *Bifidobacteria* and *Lactobacilli*, which are common species of probiotics existing in most people, can affect immune function by various pathways. In many cases, probiotics supplementation was demonstrated to induce TGF- β expression, which ameliorates food allergy by suppressing Th2 response, and inducing Foxp3⁺ Treg production [10-15]. A microarray analysis of intestinal epithelial cells from gnotobiotic mice revealed a mechanism that *Clostridia* facilitated immune cells to produce interleukin-22 (IL-22), regulated innate lymphoid cell function and intestinal epithelial permeability to protect against allergen sensitization [3]. Besides, the suppressive effect of probiotics on Th17 response has been shown both in murine asthma [16] and atopic dermatitis model [17]. However, whether probiotics treatment elicited changes in the composition of the intestinal microbiota, thereby regulating allergic disease remains poorly understood.

The current study investigated the beneficial effect of *Bifidobacterium Infantis* (BB) in a

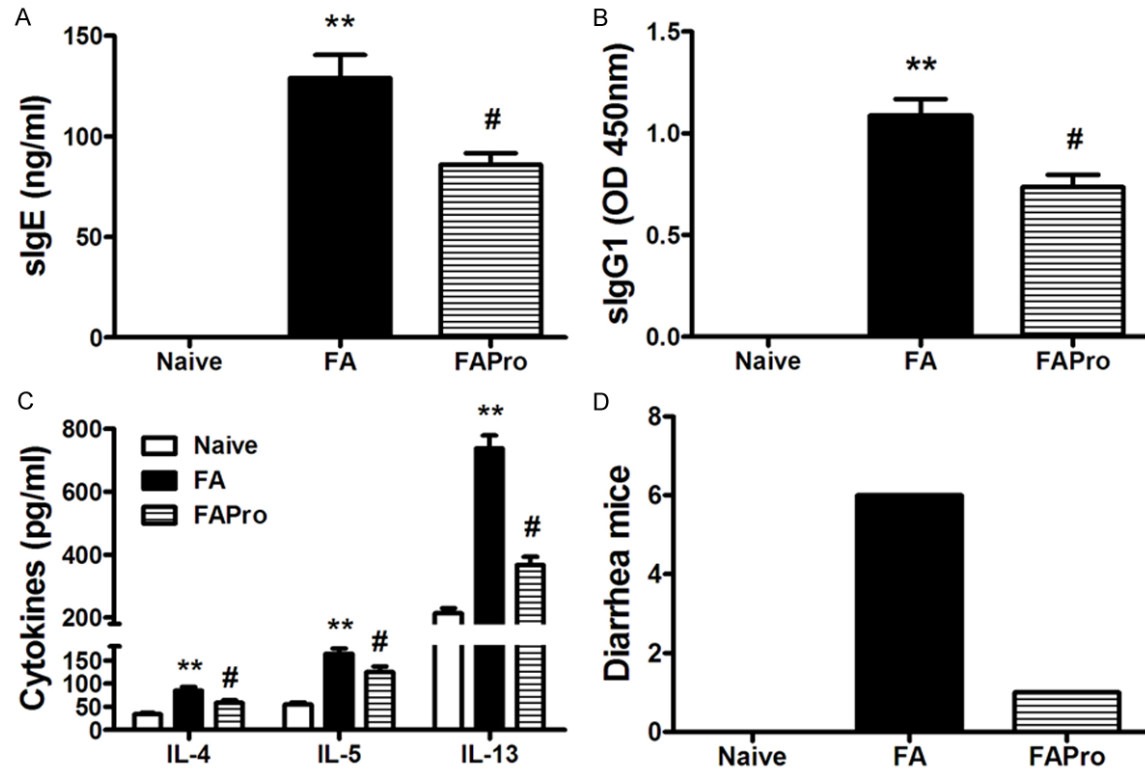


Figure 1. Allergic reactions in the mouse intestine were attenuated by BB. Balb/c mice were treated with PBS (Naïve group), OVA/CT (FA group), OVA/CT+BB (FAPro group). The bars indicate the levels of serum OVA-specific IgE (A), -IgG1 (B), IL-4, -5, and -13 levels in supernatant of splenocytes (C), and number of diarrhea mice (D). Data are presented as mean ± SEM. ** $P < 0.01$ vs naive group; # $P < 0.05$, vs FA group.

murine model of food allergy at the level of commensal microbiota. Sequencing of the V4-V5 regions of 16S rRNA genes revealed that BB could modulate specific genera of intestinal microbiota in mice, which may induce immune responses in gastrointestinal tract to defend against food allergens.

Materials and methods

Animals

All the animal experimental procedures were conducted according to the guidelines approved by the Experimental Animal Ethic Committee at Shenzhen University, and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). 6-8 weeks old female Balb/c mice were housed in a SPF animal facility with a 12 h light-dark cycle and were free to access standard diet and water.

Food allergic animal model

Mice were intragastrically administered with 100 mg OVA plus 20 mg cholera toxin (CT) in a final volume of 300 µl using a ball-end mouse feeding tube once a week for 4 consecutive weeks. At the end of sensitization, mice were challenged with 5 mg OVA orally. After 24 h, the mice were killed and serum and splenocytes were collected for the following analysis as reported previously (referred to as FA group) [25].

BB preparation and supplementation

BB was kindly provided by Shenzhen Kexing Biotech CO., LTD (Shenzhen, China) as lyophilized powder and inoculated before giving to mice. From Day 15 to Day 28, sensitized mice were orally administered with 200 µl/mouse of normal saline containing 10^8 cfu/ml as previously described (referred to as FAPro group) [13]. On day 29, the mice were challenged as described above.

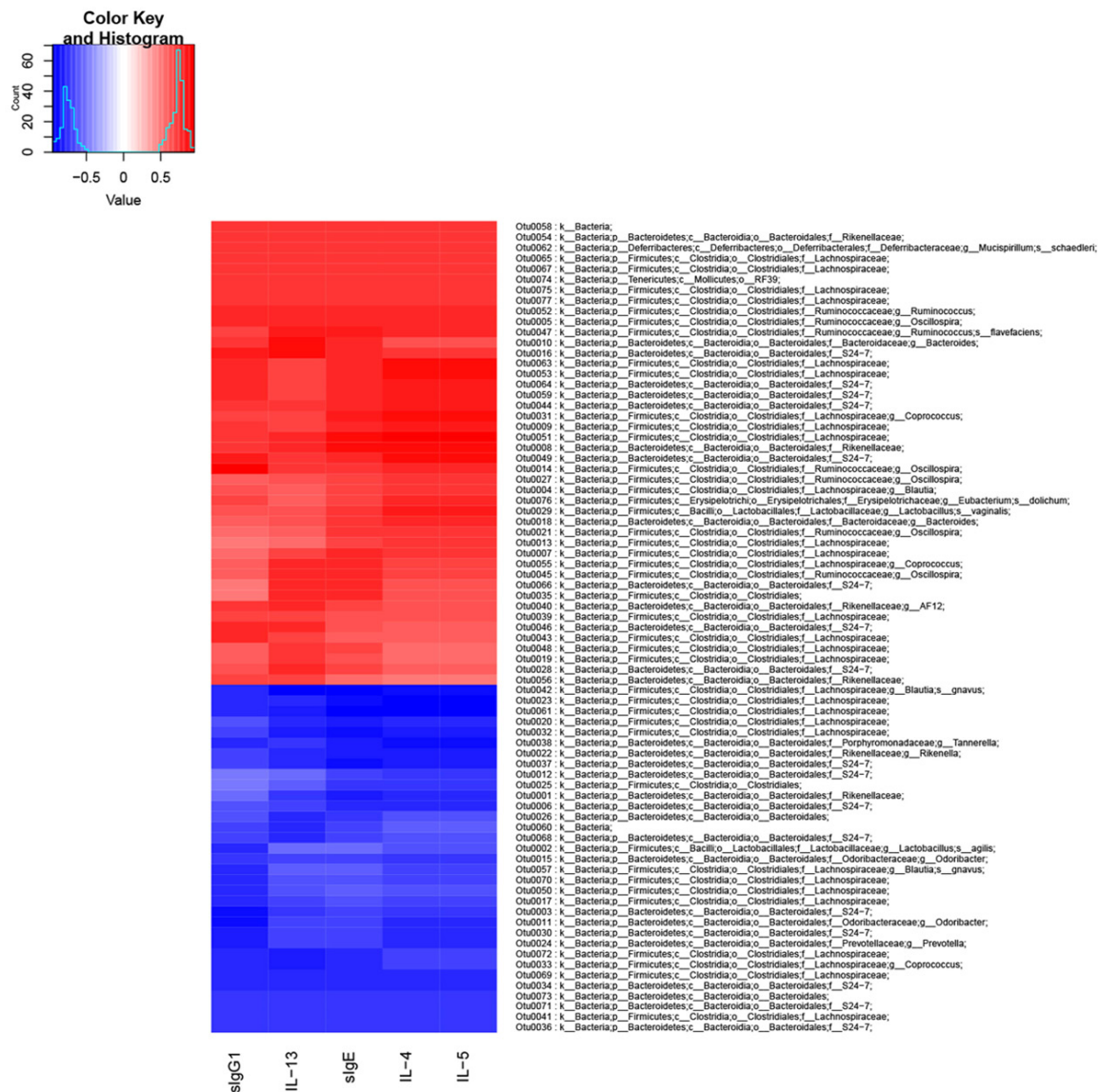


Figure 2. The heatmap of correlation between five phenotypes and OTUs profile. Red means positive correlation, while blue represents negative correlation.

Serum immunoglobulin levels

Serum was collected, and OVA-specific IgE was detected by commercial ELISA kit (Biolegend, USA) according to the manufacturer's instructions. OVA-specific IgG1 was measured by an in-house ELISA as previously described [26].

DNA extraction, amplification and sequencing

During the process of food allergy model establishment, fecal samples (up to ~1 g) were collected on Day 0, 7, 14, 28, 29, and stored at -80°C. The total DNA from fecal samples was extracted by reported method [27]. The 16S

rRNA was amplified and sequenced on the Ion Torrent Personal Genome Machine as reported in previous study [28].

Bioinformatics analysis

The data was treated with in-house pipeline developed based on mothur v.1.33.3 [29]. The community structure was calculated based on the membership and relative abundance of taxonomic groups in the sample. In this study, the Permutational multivariate analysis of variance (PERMANOVA) was used to assess the effect of BB (covariate) on operational taxonomic units (OTUs) profiles. A two-tailed

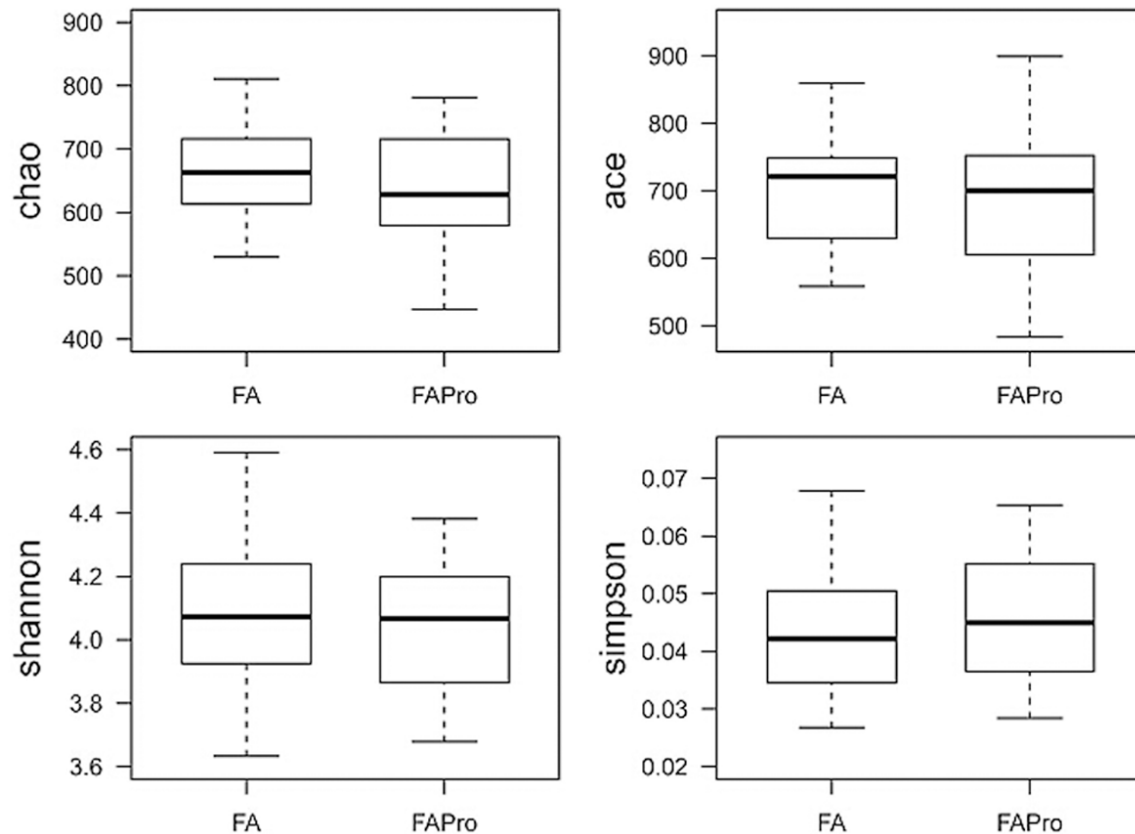


Figure 3. Boxplot of 4 kinds of alpha diversity between FA and FAPro group. Chao, ACE, Shannon, simpson are the four kinds of alpha diversity metrics. FA (n=27), FAPro (n=34). Mean values $\pm$ SEM are plotted.

Wilcoxon rank-sum test was used in the profile to identify the different OTUs and KEGG Orthologs (KOs). In addition, we used PICRUST [30] to produce predicted KOs from the 16S rRNA gene sequence data.

Statistical analysis

In **Figure 1**, all values are presented as the means $\pm$ SEM. Differences between two groups were evaluated with the Student t test, while data among three or more groups were evaluated with one-way ANOVA (Prism version 5, GraphPad Software; CA, USA). A *P* value less than 0.05 was considered to indicate significant differences.

Results

BB showed significant protective effect on food allergic mice

Food allergic mice model was established using OVA as allergen, CT as adjuvant. As shown in

Figure 1A and **1B**, treatment with BB for two weeks attenuated sIgE and sIgG1 by 33% and 32% respectively, when compared with FA group. Moreover, splenocytes were harvested from all the three groups of mice and incubated with OVA for 3 days. The levels of typical Th2-type cytokines in supernatant were determined by commercial ELISA. Intragastrically administered with BB significantly reduced IL-4, -5, and -13 by 31%, 24%, and 50% respectively in FA mice (**Figure 1C**). In addition, after challenge with OVA, the FA mice showed significant diarrhea (**Figure 1D**), which could be ameliorated by BB.

BB-induced phenotypic improvement was associated with specific OTUs

Next, to investigate the effect of BB on gut microbiome, we carried out metagenomic sequencing of fecal samples from FA and FAPro mice. All sequencing reads were finally classified into 1195 operational taxonomic units

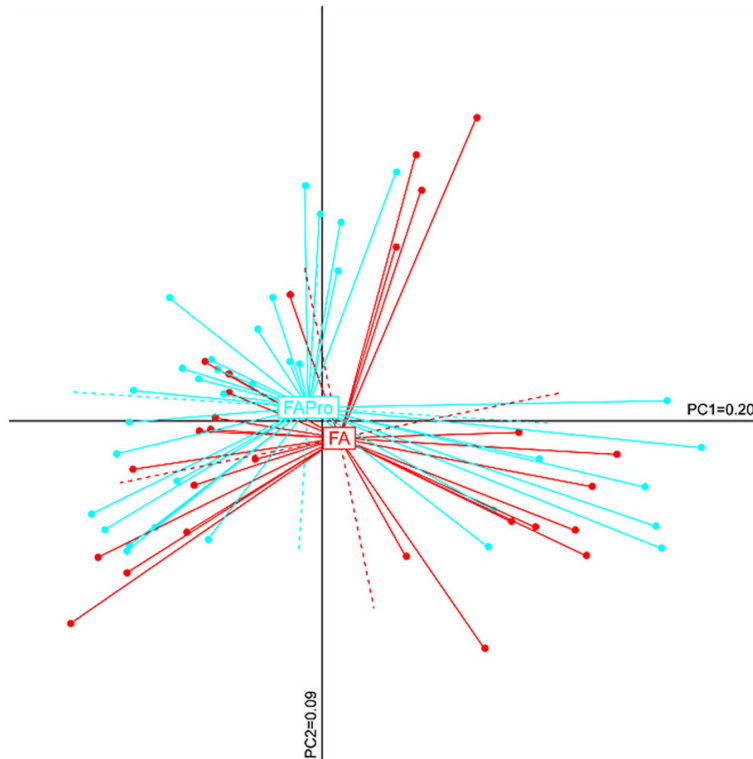


Figure 4. The PCoA of OTU profile between FA and FAPro mice. 16S rRNA gene surveys (analyzed by JSD-based PCoA) from mice fed PBS (red) or probiotics (blue) diets are presented in a different clustering pattern. Principal coordinate1 (PC)1 and PC2 are the x axis and y axis, respectively, and have been scaled on the basis of percent variance.

(OTUs). The correlation between food allergic phenotypes and OTUs was calculated. It was found that 61 OTUs were significantly related to *slgE*, *slgG1*, *IL-4*, *IL-5*, and *IL-13*. Among them, 45 OTUs were positively correlated with these phenotypes and 16 OTUs were negatively correlated (**Figure 2**). For instance, *Otu0724*, annotated to the family *S24-7*, was significantly positive correlated with allergic phenotypes. On the contrary, *Otu0543*, annotated to the genus *Bacteroides*, was significantly negatively correlated. Upregulation or downregulation of the relative abundances of these OTUs could trigger certain immune responses. The results indicated that BB treatment may change immune indexes of food allergy through modulation of these OTUs.

Treatment with BB shows no effect on alpha-diversity of intestinal microflora

Chao [18] and ACE [19] are usually used to compute community richness; the higher score, the more richness. Shannon and Simpson metrics are commonly used to calculate community

diversity [20]. The higher Shannon index indicates the greater community diversity, while the higher Simpson index indicates the lesser community diversity. We used these 4 kinds of alpha diversity parameters to describe the microbiologic species diversity changes between FA group and FAPro group (**Figure 3**). Student's t-test showed that there were no significant differences of these four indexes (**Figure 3**). The results indicated that BB was not strong enough to change population diversity and richness of intestinal microbiota.

BB didn't alter intestinal microbiota composition in mice

In order to investigate whether probiotics treatment change the composition of intestinal microbiota, we used principal coordinate analysis (PCoA) to compare FA and FAPro group. As shown in **Figure 4**, there was no significant

difference between FA and FAPro group. Thus, it was implied that BB showed no effect on modulation of microbiota composition.

The taxonomic classification of gut microbiota in mice

We found that *Bacteroidetes* and *Firmicutes* were two most prevalent phyla present in food allergic mice treated with or without probiotics, the same as that under physiological status [3]. Furthermore, *Lachnospiraceae*, *S24-7*, *Rikenellaceae*, and *Ruminococcaceae* accounted for four major components at family levels (**Figure 5A**). Further analysis revealed that 2-wk of BB treatment resulted in a significant change in fecal microbiota composition at genus level. As shown in **Figure 5B**, the levels of *Coprococcus* and *Rikenella* were significantly increased by 66% and 60% respectively, after BB treatment. Thus, the relative abundances of *Coprococcus* and *Rikenella* may be used as microbial biomarkers to diagnose food allergy.

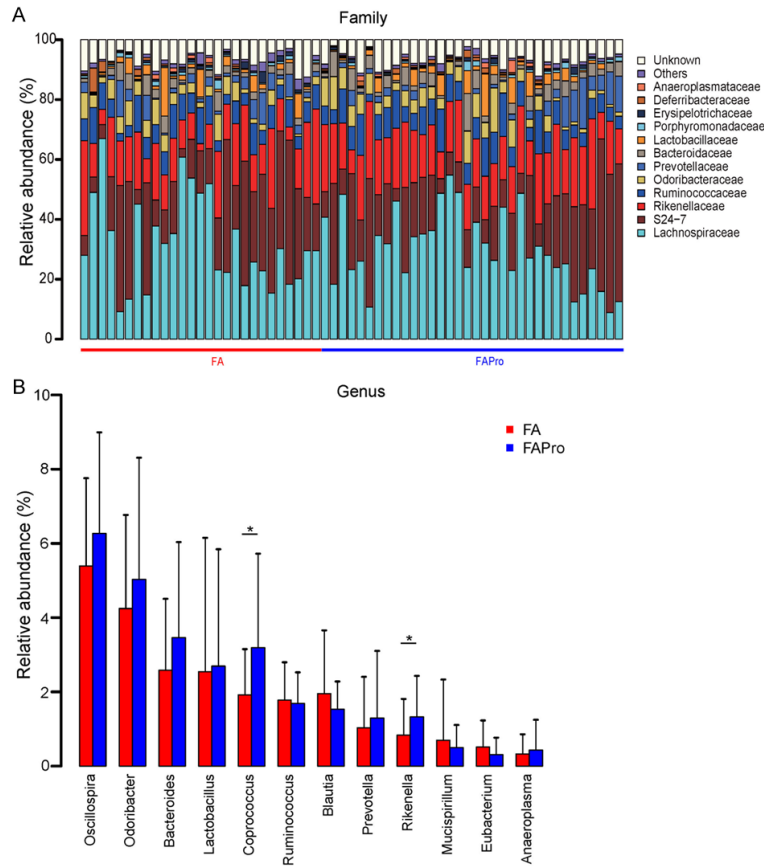


Figure 5. A. Taxonomic distributions in gut communities. Values represent the relative abundance of bacteria at family level across all samples within FA group and FAPro group. A small amount of microorganism is unknown. B. Comparison of 12 major genera between FA and FAPro group. Wilcoxon rank test showed there were significant differences in *Coprococcus* and *Rikenella* between the two group. * $P < 0.05$, ** $P < 0.01$ vs FA group.

Comparison of OTUs levels between FA and FAPro mice

Next, Wilcoxon rank test showed that 92 OTUs were significantly different between FA group and FAPro group. Among them, 40 OTUs (43.5%) were enriched in FA group. 33 OTUs were picked out through a FDR adjust and make a heatmap with the OTU percentage profile (**Figure 6**). Moreover, we found that probiotics administration could enrich more bacteria assigned to *Coprococcus*, *Rikenella* and *Bacteroides* in the mice gut (**Figure 6**).

Mice gut microflora changed across time

In order to monitor the change of gut bacteria during the period of probiotics administration, we collected fecal samples at 5 time points: before oral treatment of probiotics (FAPro1), after one week's probiotics administration

(FAPro2), after two weeks' administration (FAPro3), 1 h after allergen challenge (FAPro4), 24 h after allergen challenge (FAPro5). Intriguingly, we selected 12 most abundant genera and found that at least 6 genera of gut bacteria, including *Odoribacter*, *Bacteroides*, *Coprococcus*, *Blautia*, *Eubacterium*, *Prevotella* changed with time after probiotics treatment (**Figure 7**). For example, the levels of *Odoribacter* were significantly increased by 3.3 fold at the time point of 24 h after challenge compared to the time point of 1 h after challenge.

Metabolic pathways of gut microbiota was altered by BB supplementation

We used PICRUST to produce predicted metagenomes from 16S rRNA gene sequence database. 143 KOs were found to be significantly different between FA and FAPro mice, using Wilcoxon rank test, p value < 0.05 . Among them, only 4 KOs were enriched in FAPro group (**Table**

1). The results implied that BB supplementation significantly modified metabolic pathways of gut microbiota.

Discussion

Gut microbiota plays an important role in the pathogenesis of food allergy. In this study, we found that oral administration of BB induced significant improvement on allergic symptoms in mice. Furthermore, the results demonstrated that BB conferred a protective effect on food allergic mice through up-regulation of the relative abundance of *Coprococcus* and *Rikenella* at genus level. Furthermore, the genera of gut microflora were presented in a time-dependent pattern after BB treatment.

Growing evidence suggests that the relationship among diet, probiotics, immune system and gut microbiota ecology determines the dis-

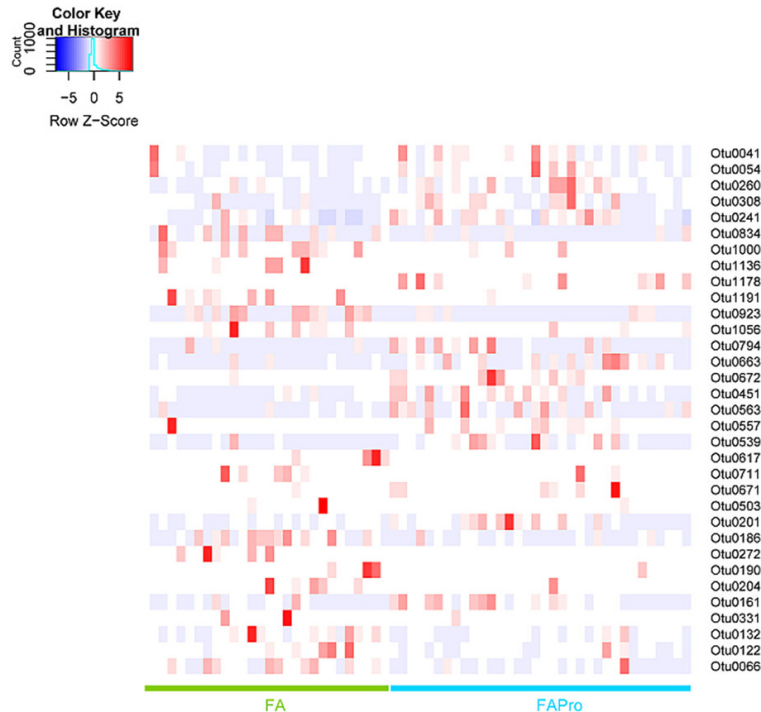


Figure 6. Heatmap of gut bacteria in FA and FAPro group at OTU level. Blue regions represent relatively low OUT abundance, while red regions means relatively high OTU abundance.

ease susceptibility to allergy [21]. Thus, it is very likely that intragastrical administration of probiotics may treat food allergy by restoring the unbalanced indigenous microbiota and controlling the inflammatory responses. Until now, there is no investigation targeting the direct effect of probiotic supplementation on intestinal microbiota. Although there are more than 1000 species of intestinal bacteria, most of them belong to just a few phyla. *Bacteroidetes* and *Firmicutes* phyla dominate the adult intestine. The intestinal microbiota is of high variation from people to people at species-level, but *bifidobacteria* and *lactobacilli* are common species existing in most people [22]. Thus, in the present study we chose BB to treat a classical animal model sensitized by OVA. In this study, animals treated with probiotics for two weeks showed improvement in all major indicators of experimental mucosal allergy, in line with the results previously reported [23].

When use traditional culture based techniques to determine the composition of the gut microbiota, there are only ~10% of gut bacteria possible to be studied since others are not cultur-

able [24]. Therefore, in order to further determine the different components of intestinal microbiota caused by probiotics, we chose state-of-the-art next-generation sequencing method to detect the 16S rRNA of feces samples and determine the frequency of microbes and its metabolic pathway in gastrointestinal tract. We found that there were 12 genera of gut bacteria existing in both FA and FAPro groups. After supplementation with BB for two weeks, each genus changed periodically. Based on their relative abundances, BB administration could up-regulate *Rikenella* and down-regulate *Eubacterium*. These two genera of bacteria have never been highlighted by other related researches. Instead, Stefká [3] *et al* demonstrated that a *Clostridia*-containing microbiota was associated with innate lymphoid cell function and intestinal epithelial permeability. The divergence may be attributed to that they didn't use a kind of probiotics to treat allergic mice.

In conclusion, this is the first study to explore microbial population changes in food allergic animal model, in case of probiotics administration. Likely, specific gut bacterial changes contributed to disease process altered by probiotics. Still, patients study are warranted in the future to determine whether the findings herein reported can be validated and correlated with the clinical features.

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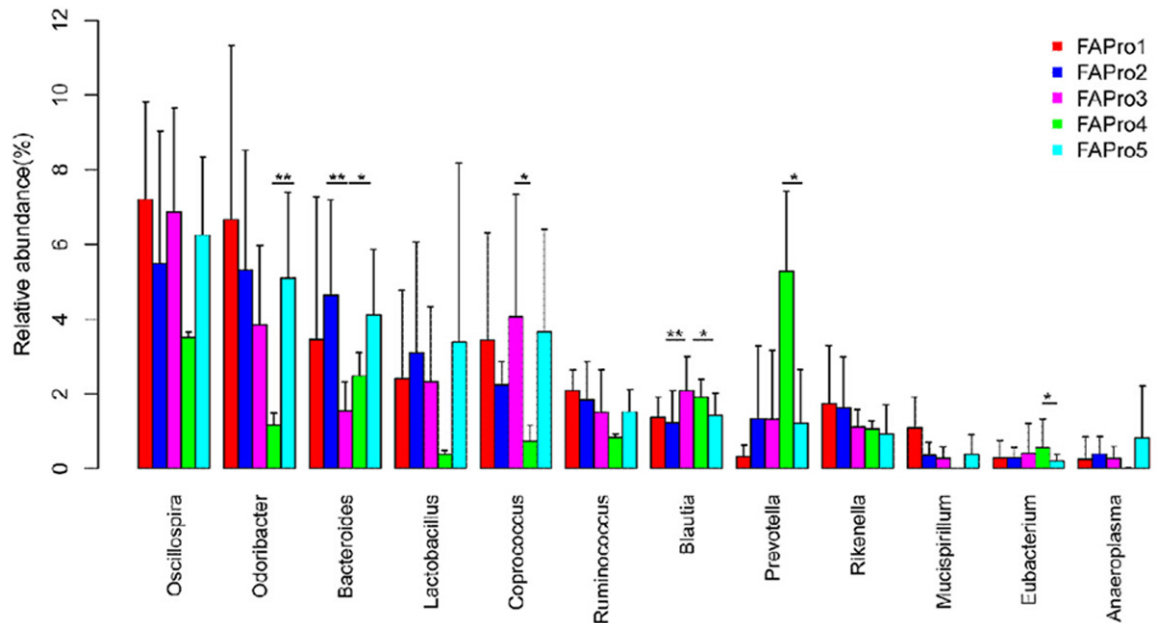


Figure 7. Time-dependent manner of gut bacteria changes at genus level. During the period of probiotics administration, we collected fecal samples at 5 time points: before oral treatment of probiotics (FAPro1), after one week's probiotics administration (FAPro2), after two weeks' administration (FAPro3), 1 h after allergen challenge (FAPro4), 24 h after allergen challenge (FAPro5). The relative abundances of bacteria at genus level are presented. * $P < 0.05$, ** $P < 0.01$, PAPro2 vs PPro1, PPro3 vs PPro2, PPro4 vs PPro3, PPro5 vs PPro4.

Table 1. Four KEGG Orthologs were enriched in FAPro group

	FAPro Mean	FA Mean	FAPro SD	FA SD	multiple	<i>p</i> value*	KEGG description
K13820	1.970588235	0.222222	3.745586	0.423659	8.867647	0.002142	Flagellar biosynthetic protein FliR/FliB
K09612	1.970588235	0.222222	3.745586	0.423659	8.867647	0.002142	Alkaline phosphatase isozyme conversion protein [EC:3.4.11.-]
K06956	3.764705882	3.62963	4.465754	8.321188	1.037215	0.013217	None
K11693	2.5	1.666667	3.963852	5.053559	1.5	0.014967	Peptidoglycan pentaglycine glycine transferase (the first glycine) [EC:2.3.2.-]

**P* value was calculated by Wilcoxon rank test.

Disclosure of conflict of interest

None.

Authors' contribution

B.Y., L.X. and S.L. performed experiments and analyzed data. B.Y. wrote the manuscript. X.Y.L. and Y.L. performed experiments. Q.M.J, P.C.Y. and Z.G.L. organized the project and supervised the experiments. P.C.Y. revised the manuscript.

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